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A cAMP receptor-like G protein-coupled receptor with roles in growth regulation and development

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Abstract

Dictyostelium discoideum uses G protein-mediated signal transduction for many vegetative and developmental functions, suggesting the existence of G protein-coupled receptors (GPCRs) other than the four known cyclic adenosine monophosphate (cAMP) receptors (cAR1–4). Sequences of the cAMP receptors were used to identify *Dictyostelium* genes encoding cAMP receptor-like proteins, CrlA–C. Limited sequence identity between these putative GPCRs and the cAMP receptors suggests the Crl receptors are unlikely to be receptors for cAMP. The *crl* genes are expressed at various times during growth and the developmental life cycle. Disruption of individual *crl* genes did not impair chemotactic responses to folic acid or cAMP or alter cAMP-dependent aggregation. However, *crlA*$^-$ mutants grew to a higher cell density than did wild-type cells and high-copy-number *crlA* expression vectors were detrimental to cell viability, suggesting that CrlA is a negative regulator of cell growth. In addition, *crlA*$^-$ mutants produce large aggregates with delayed anterior tip formation indicating a role for the CrlA receptor in the development of the anterior prestalk cell region. The scarcity of GFP-expressing *crlA*$^-$ mutants in the anterior prestalk cell region of chimeric organisms supports a cell-autonomous role for the CrlA receptor in prestalk cell differentiation.

Keywords: G protein-coupled receptor; cAMP receptors; *Dictyostelium*; Development; Cell density

Introduction

Current understanding of G protein-coupled receptor (GPCR) signal transduction is attributed to the analysis of receptors from a wide range of organisms, including genetically accessible organisms such as *Dictyostelium discoideum*. The dynamic amoeboid cell movement and relatively simple developmental life cycle of *Dictyostelium* allow for the assignment of many signal transduction components to particular chemotactic and developmental processes in this organism (Firtel, 1996; Parent and Devreotes, 1999). *Dictyostelium* migration to bacterial sources, transition from unicellular growth to multicellular development, morphogenesis of the aggregate into the mature fruiting body, and differentiation of prespore and prestalk cells are all influenced by G protein-mediated signal transduction (Hadwiger and Firtel, 1992; Hadwiger et al., 1994, 1996; Kumagai et al., 1991; Louis et al., 1994; Saxe et al., 1993; Sun and Devreotes, 1991).

The genetic and biochemical characterization of four cyclic adenosine monophosphate (cAMP) receptors (cARs) and other signaling components in *Dictyostelium* has thus far provided important insights into the mechanisms of gradient sensing during chemotaxis, glycogen synthase kinase 3 (GSK3)-mediated cellular differentiation, and novel G protein-independent modes of GPCR signaling (Brzostowski and Kimmel, 2001; Hereld and Devreotes, 1992; Janetopoulos et al., 2001; Kim and Kimmel, 2000; Kim et al., 1997; Parent and Devreotes, 1999). The cARs are known to function during distinct developmental stages and in different subsets of developing cells within multicellular aggregates, indicating multiple roles for secreted cAMP signaling (Briscoe et al., 2001; Dormann et al., 2001; Hereld and Devreotes, 1992; Johnson et al., 1992, 1993; Louis et al., 1994; Saxe et al., 1993, 1996; Sun and Devreotes, 1991; Yu and Saxe, 1996).

Abbreviations: cAMP, cyclic adenosine monophosphate; GPCR, G protein-coupled receptor; GSK3, glycogen synthase kinase 3; LPA, lysophosphatidic acid.

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Several lines of evidence suggest *Dictyostelium* possesses additional GPCRs. First, this organism's G proteins, including nine characterized G protein α-subunits and several others emerging from cDNA and genome sequencing projects, greatly outnumber the cARs (Brandon et al., 1997; Brzostowski et al., 2002; Hadwiger et al., 1991; Pupillo et al., 1989; Wu and Devreotes, 1991). In many organisms, the number of receptors often exceeds the number of G proteins and this consequently results in different receptors coupling to common G proteins. In *Dictyostelium*, at least three and possibly all four cARs can couple with the Gα2 G protein and none appear to couple with other G protein subunits based on phenotypic differences between receptor and G protein knockout mutants (Brandon et al., 1997; Hadwiger and Firtel, 1992; Hadwiger et al., 1996; Kim et al., 1998; Kumagai et al., 1991; Louis et al., 1994; Saxe et al., 1993; Wu et al., 1994). Second, several other external signals [e.g., folate, lysophosphatidic acid (LPA), CMF, PSF, CF], for which receptors have yet to be identified, are believed to act through G proteins (Clarke et al., 1992; Gomer et al., 1991; Hadwiger and Srinivasan, 1999; Jalink et al., 1993; Okuwa et al., 2001; Roisin-Bouffay et al., 2000; Sordano et al., 1993). Third, defects in phagocytosis and slug motility associated with loss of the only known *Dictyostelium* G protein β-subunit suggest roles for novel GPCRs in these processes (Milne et al., 1995, 1997). Therefore, we culled the accruing sequence data of the *Dictyostelium* cDNA and genome projects and identified several GPCR candidates, anticipating the analysis of these putative receptors will define *Dictyostelium* signaling pathways and yield paradigms for GPCR function and regulation in higher organisms.

Here, we report the identification and characterization of three cAMP receptor-like putative GPCRs designated CrlA–C. Although they are most closely related to the cARs, they differ substantially from them and one another, suggesting that the Crls represent one or more new families of *Dictyostelium* receptors. While all three Crl receptors are expressed during development, only one appears to be required for normal cell growth and developmental morphogenesis. We demonstrate that CrlA functions cell-autonomously in cell growth regulation and tip formation in developing aggregates.

Materials and methods

Strains and media

Disruption of the *crlA* gene was performed in the axenic strain KAx-3 and the disruptions of the *crlB* and *crlC* receptor genes were conducted in the axenic strain Ax-3. In the case of the *crlC* receptor, a gene disruption was also created in the strain Ax-2 because of potential instability of the gene disruption in the Ax-3 strain. All phenotypic analyses were performed using the appropriate parental strains (KAx-3, Ax-3, or Ax-2) as a control. *Dictyostelium* cells were grown axenically in HL-5 medium or on bacterial lawns of *Klebsiella aerogenes*. Cells were transformed by electroporation and transformants were selected in HL-5 medium containing the appropriate drug (G418 or blasticidin) as previously described (Natarajan et al., 2000). In the chimeric studies, cells were labeled with GFP (green fluorescent protein) expression by introducing the vector pTX-GFP as previously described (Levi et al., 2000).

Recombinant DNA methods

Sequence information, genomic fragments, and cDNAs of the *crlA–C* genes were obtained through the *Dictyostelium* Developmental cDNA Project (Morio et al., 1998), the Genome Sequencing Centre Jena—*Dictyostelium* genome project (website at http://genome.imb-jena.de/dictyostelium/), and the Baylor Sequencing Center—*Dictyostelium* genome project (website at http://dictygenome.bcm.tmc.edu/). Additional sequencing was performed in sequencing facilities at Oklahoma State University and University of Texas Medical School—Houston. The *crlA*, *crlB*, and *crlC* GenBank accession numbers are AY219179, AY360136, and AY360135, respectively. Genomic DNA blots and RNA blots were conducted as previously described (Hadwiger et al., 1996) using radioactive probes generated by the random primer method (Feinberg and Vogelstein, 1983).

For reverse transcription-polymerase chain reaction (RT-PCR) analysis of gene expression, wild-type Ax-3 cells were developed on agar plates and total RNA was isolated every 4 h until fruiting bodies were formed using Trizol reagent (GibcoBRL). The RNA was then treated with DNase, phenol-extracted, precipitated, and quantified by absorbance at 260 nm. For reverse transcription, RNA (2 μg) and random hexanucleotides (0.5 μg) were combined in 14 μl, denatured at 70 °C for 5 min, and chilled on ice for 5 min. AMV reverse transcriptase (9 U), buffer, and dNTPs (0.4 μM each) were then added and the volume was adjusted to 25 μl. Reactions were incubated 10 min at 20 °C followed by 50 min at 42 °C. The resulting cDNA was then amplified by PCR using specific primers. PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide.

A contiguous *crlA* gene was created from sequencing vectors by replacing a *Hin*dIII–*Spe*I fragment of sequencing vector pJC2d106a03 with a more complete *Hin*dIII–*Spe*I *crlA* upstream fragment from vector pIIBCP1D0339 to produce vector pBR6. Gene disruptions at the *Spe*I site were created by inserting the Bs^r (blasticidin S resistance) gene in both orientations as an *Xba*I fragment from the vector pJH380, a derivative of pBsr2 (Sutoh, 1993). Individual $crlA^-$ mutants were created using either gene disruption construct. Only one strain was used for most of the analyses since both mutants displayed similar phenotypes. Genomic DNA blots were used to verify gene disruptions. The *crlA* expression vector, pBR16, was created by insert-

ing the upstream *Hin*dIII–*Spe*I fragment of pBR06 into the same sites of vector pBR11 that contained a G418 resistance gene inserted into the *Eco*RI–*Bgl*II sites of pJC2d106a03. This construct contains approximately 400 bp of sequence upstream of the *crlA* coding region. A *crlA*^{fs} frameshift mutation was created in pBR16 at the unique *Spe*I site in the *crlA* coding region by using Klenow DNA polymerase to fill in the overhangs of the *Spe*I site before re-ligation. The resulting *crlA*^{fs} vector was designated as pBR28.

For disruption of the *crlB* gene, a 1.4-kb gene fragment was amplified using primers ATcCAcCTTTGTTCAATGATTTTA and ATAGAATCTAGTTGTAACAGCATA (lowercase nucleotides differ from those in gene) and blunt-end ligated into the *Sma*I site of pBluescript. The *Bs*^{r} gene was then moved as a *Pst*I fragment from pBSR519 (Puta and Zeng, 1998) and inserted into a unique *Nsi*I site near the *crlB* fragment's midpoint to yield pMZ52. The knockout construct was released by *Eco*RI and *Not*I digestion and transformed into Ax-3 cells.

For *crlC* gene disruption, an approximately 0.8-kb 5′-fragment of the *crlC* gene was amplified from a pBluescript(KS^{-}) sublibrary of approximately 5-kb *Hin*dIII–*Bgl*II genomic fragments using a *crlC* antisense primer tcggatccAGgGAAGATTGGTGGAGGTTGG, which introduces a *Bam*HI site, and a T3 primer. The *Bs*^{r} gene, derived from pBSR519 by *Bam*HI digestion, was blunt-end ligated into the *Sma*I site of pBluescript, yielding pBSR52 in which an upstream *Pst*I site and a downstream *Bam*HI site flank the *Bs*^{r} gene. A 3′ *crlC* gene fragment was amplified using a *Pst*I-tagged sense primer (tactgcagGATCGTGAATTTTTGTCAGTTGAC) and an antisense primer (GAATTACATCTGGTAACCAAC) downstream from a natural *Xba*I site. The *crlC* knockout construct was then assembled from these components in a four-piece ligation containing: (1) the 5′ *crlC* fragment digested with *Hin*dIII and *Bam*HI; (2) the *Bs*^{r} gene released from pBSR52 with *Bam*HI and *Pst*I; (3) the 3′ *crlC* fragment digested with *Pst*I and *Xba*I; and (4) *Hin*dIII- and *Xba*I-cut pBluescript. The knockout construct was released from the resulting plasmid (pMZ37) by *Hin*dIII and *Xba*I digestion and transformed into both Ax-2 and Ax-3 cells.

For *yakA* gene disruption, the vector pSVE216, containing *yakA* flanking sequences and the *Bs*^{r} gene, was linearized at the unique *Bgl*II site and transformed into KAx-3 cells as previously described (van Es et al., 2001). Non-aggregating transformants were confirmed to be *yakA*$^{-}$ by PCR using primers that flank the site of insertion (*Dpn*II site encoding Asp358 of YakA). Besides the aggregation defect, these *yakA*$^{-}$ cells also exhibited previously described chemotaxis defects and slow plaque growth rate (van Es et al., 2001).

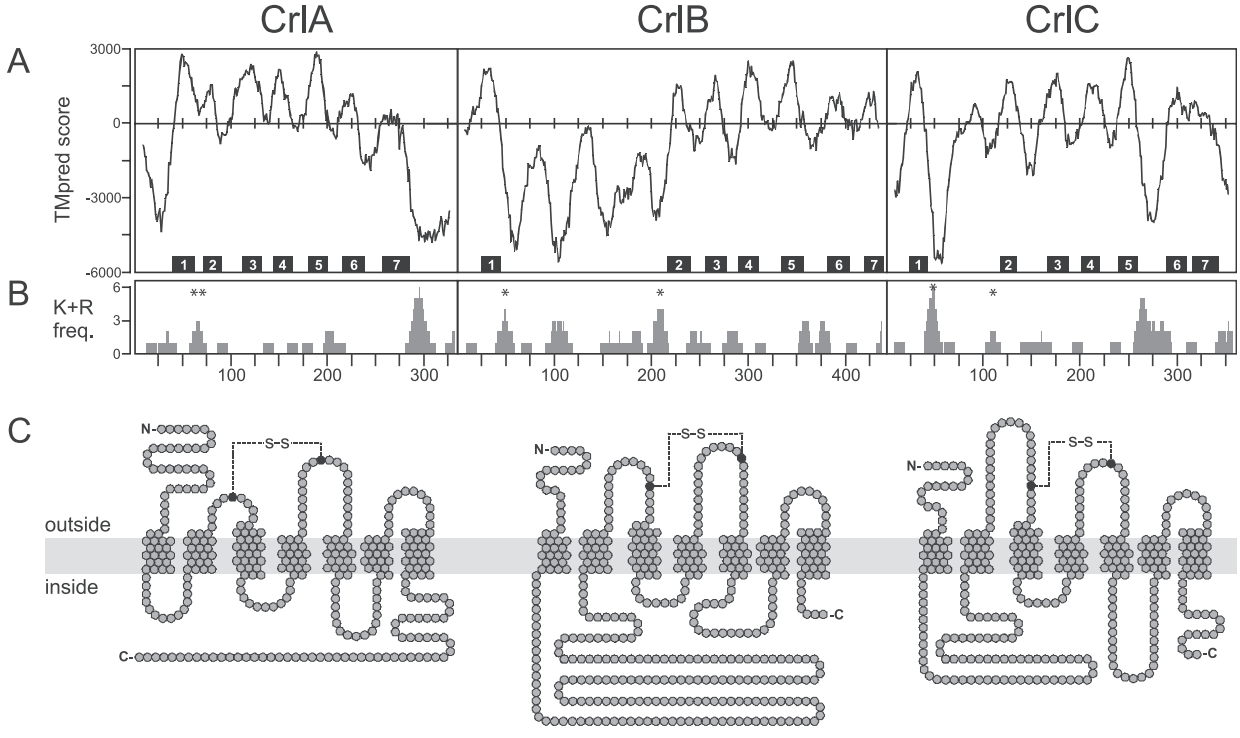
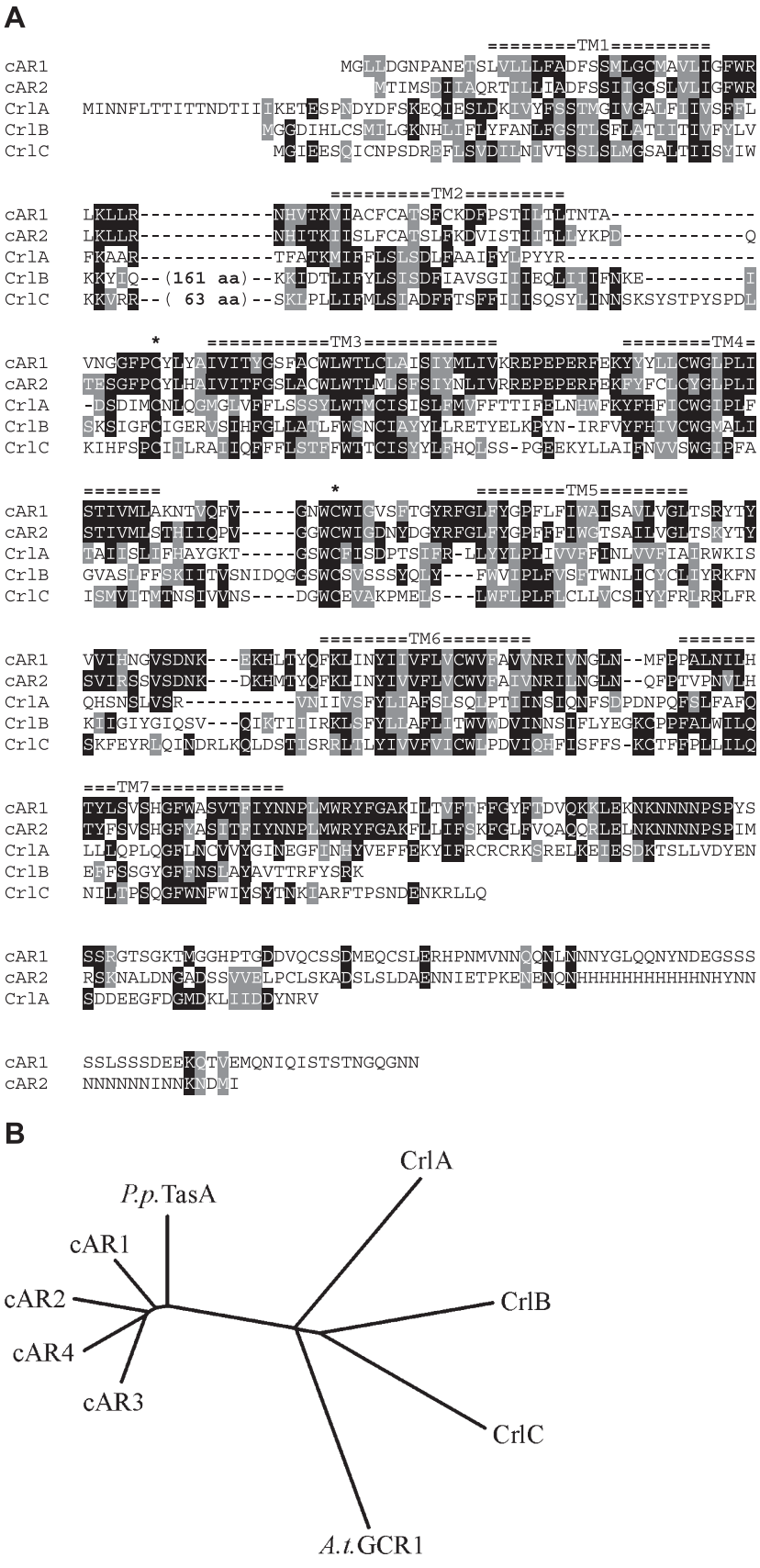


Fig. 1. Hydropathy analysis of CrlA–C. (A) The deduced amino acid sequences of CrlA–C were analyzed using the TMpred program at http://www.ch.embnet.org/. Positive scores indicate membrane spanning potential. Seven putative transmembrane domains for each are indicated by numbered black rectangles. (B) The number of lysine and arginine residues (K + R) occurring in an 11-residue window centered on each residue is plotted. The frequency of these basic residues is often elevated in the cytoplasmically disposed sequence flanking transmembrane domains (Wallin and von Heijne, 1995). The ordinate refers to amino acid residue number and pertains to both panels A and B. Asterisks (*) indicate basic regions that support the TM1 and TM2 assignments depicted in panel A. (C) Topological models for CrlA–C. Filled circles represent amino acids. Those predicted to comprise the TM domains are grouped and overly the gray bar representing the plasma membrane. Dashed lines linking conserved cysteines (black fill) are presumed to form a disulfide bridge.


A
B
CrlA
P.p.TasA
cAR1
cAR2
cAR4
cAR3
CrlB
CrlC
A.t.GCR1

Development and chemotaxis assays

Dictyostelium cells were grown to mid-log phase (approximately 2×10^6 cells/ml), washed in phosphate buffer (12 mM NaH_2PO_4, adjusted to pH 6.1 with KOH), and suspended in phosphate buffer before spotting onto non-nutrient plates (phosphate buffer, 15% agar) for development or chemotaxis assays. Analysis of developmental morphology of clonal or chimeric aggregates was conducted on non-nutrient plates spotted with 4×10^7 cells/ml suspensions. Development for RNA isolation was conducted on filter pads as previously described (Mann and Firtel, 1993). Chemotaxis assays were conducted by spotting 5 μl of cell suspension on non-nutrient plates followed by spotting of chemoattractant solutions, containing cAMP (10^{-3} M) or folic acid (10^{-2} M), approximately 2 mm away from the cell droplet as described (Hadwiger and Srinivasan, 1999). Folic acid inhibition of development assays were performed by allowing a 5-μl droplet of folic acid (10^{-2} M) to soak into a non-nutrient plate before spotting 2 μl of cell suspension at the same location on the plate.

Results

Identification and predicted structures of the Crl receptors

Dictyostelium cAMP receptor sequences were used to search *Dictyostelium* cDNA and genomic sequence databases to identify genes encoding novel receptors. Three putative GPCRs were identified and designated CrlA–C (for cAMP receptor-like) and the corresponding genes were designated *crlA–C*, respectively, in accordance with established *Dictyostelium* nomenclature. All of the Crl proteins are predicted to contain seven putative transmembrane (TM) domains typical of GPCRs (Fig. 1). For each of the Crls, assignment of TMs 3–7 was validated by homology with the corresponding TMs of the cARs (Fig. 2). Crl TMs 1 and 2, on the other hand, bear little resemblance to those of cARs. They were assigned based on hydropathy and the elevated frequency of basic residues flanking the TM domain's expected cytoplasmic end (Fig. 1B; (Wallin and von Heijne, 1995)). Thus, the assigned TM1 domains are followed by basic residues, consistent with their expected outside-to-inside orientation. Likewise, the TM2 domains, whose N-termini should be cytoplasmically oriented, are preceded by highly basic sequences. According to our models (Fig. 1C), CrlB, and, to a lesser extent, CrlC have unusually large first intracellular loops, which interconnect TM1 and TM2, as well as greatly diminished C-terminal cytoplasmic tails. Interestingly, their first intracellular loops are rich in serine and threonine residues and thus might have supplanted the C-terminal domain as the site of ligand-induced phosphorylation that regulates a wide array of GPCRs including cARs.

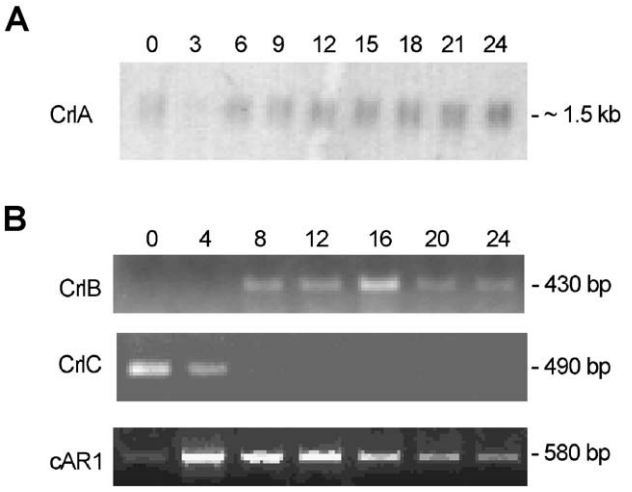


Fig. 3. Temporal expression patterns of *crlA–C* genes during development. Total RNA was isolated from vegetative (0 h) and developing cells starved for the indicated number of hours. (A) RNA blot of *crlA* transcript expression. The RNA was subjected to northern analysis using a *crlA* specific probe as described in Materials and methods. The low expression level at 3 h is an artifact of partially degraded RNA. (B) RT-PCR analysis of *crlB* and *crlC* gene expression. The total RNA was used as template for synthesis of single-stranded cDNA by reverse transcriptase as described under Materials and methods. Gene-specific PCR products were then amplified using 1.5 μl of each cDNA as template. For comparison, cAR1 was similarly analyzed except that 30-fold less cDNA template was used; comparable amounts (not shown) were saturating for cAR1, yielding maximal amounts of PCR product for all time points. Primers used are as follows: *crlB*, GGTATGGCATTGATTGGTGTTGC and ATAGAATCTAGTTGTAACAGCATA; *crlC*, GCAACCTCACACCATCCATCAG and TCAATTACAATTGGCAATGG; *cAR1*, ATGGTAGTTTTGCATGTTGG and GTAACCAAAGAATGTGAAAACTG.

Fig. 2. Comparison of Crl, cAR, and related sequences. (A) Alignment of cAR1, cAR2, and CrlA–C amino acid sequences. The BOXSHADE program was used to highlight identical residues (black background) and similar residues (gray background). The double dashed lines (====) indicate the seven predicted transmembrane domains (TMs 1–7). Conserved cysteines residues identified by asterisks (*) are predicted to form a disulfide bond in each receptor. The homologous regions of these receptors (defined below for panel B) were aligned using the ClustalW program with minor manual adjustment. The N- and C-terminal, non-homologous regions are also shown. Gaps were manually introduced in the N-terminal region for alignment of predicted TM1 domains. The N-terminus of CrlA might extend an additional 34 residues (MIQILLSTFISFIIIIVSSNDIRSGENDNFNNNK) if a potential upstream start codon is utilized. For clarity, non-homologous sequences between TM1 and TM2 were omitted from CrlB (NKSFRENPHQYCHQHQYFDSSKLNEINNSGVGSYSSTPISIQNNNNKNNNLPKQKNNEKQPLINKNHNNYCNYSTSATSSSSSSSSFSSTNSGSSYEYQQPQKNQQTLSSSDKNNTIPSTNTKYEIELSIPQFKGNKCGPNCLLFSNIPQIKNALEQKKNP) and CrlC (HRIQKQQIQQQQQQIEKGGLLSSSITIGNGSSHYGGIGGGGGSGNGTGIGAIGGPHGTYKQPT). (B) Unrooted phylogenetic tree of CrlA–C and cAR1-4. Also included are cAMP receptor homologs of *P. pallidum* (*P.p.*TasA; GenBank accession AB045712) and *A. thaliana* (*A.t.*GCR1; GenBank accession U95142). The dendrogram was generated at http://clustalw.genome.ad.jp/ by the neighbor-joining method from a ClustalW alignment of the homologous region of each receptor. This region, spanning TM2 through TM7, includes the following amino acids: cAR1, 43–267; cAR2, 40–266; cAR3, 53–278; cAR4, 40–268; TasA, 61–285; CrlA, 66–279; CrlB, 211–442; CrlC, 112–350; and GCR1, 49–279.

Within the highly conserved regions encompassing the transmembrane domains, the CrlA receptor is 19% identical to the cAR1 and cAR2 receptors whereas CrlB and CrlC receptors share 13% and 11% identity with these cAR receptors as indicated by protein alignments and dendogram analysis, suggesting the CrlA receptor is more related to the cAR receptor family (Fig. 2). However, the cAR receptors share much more identity with each other (approximately 70% between each pair of cARs) and also with TasA, a potential cAMP receptor in the related species *Polysphondylium pallidum*, suggesting the Crl receptors might represent new outlying members of the GPCR family F (Kolakowski's classification) that includes the cAR and TasA receptors (Josefsson, 1999; Kawabe et al., 2002; Kolakowski, 1994). Another related member of this GPCR family is the *Arabidopsis thaliana* GCR1 receptor that plays a role in plant growth and development through cytokinin signal transduction (Colucci et al., 2002; Josefsson and Rask, 1997; Plakidou-Dymock et al., 1998).

A feature common to virtually all GPCRs is a pair of conserved cysteines (one in each of the first and second extracellular loops) that have been shown to form a functionally important disulfide in a variety of GPCRs by biochemical criteria, mutagenesis, and X-ray crystallography (Palczewski et al., 2000; Strader et al., 1994). Interestingly, all of the cAR and Crl receptors also have conserved cysteines in these loops (marked by asterisks in Fig. 2), which presumably form a disulfide linkage. We have preliminary evidence for such a disulfide in the cAR1 receptor (M. Goswami and D. Hereld, unpublished observation).

Expression of Crl genes

The temporal expression patterns of the *crlA–C* genes were examined in growing and developing cells to determine when the receptors might function. The *crlA* gene is expressed during vegetative growth and throughout development, as determined by RNA blot analysis, suggesting this receptor might function in either phase (Fig. 3A). The transcript for the *crlA* gene was difficult to detect relative to that of other developmentally expressed genes implying a low level of receptor expression. Expression of the *crlB* and *crlC* genes was assessed by RT-PCR (Fig. 3B). Unlike *crlA*, both *crlB* and *crlC* genes were found to be highly regulated in their expression. The *crlB* transcript was only detected in post-aggregative developmental stages, peaking after 16 h of starvation. In contrast, the *crlC* transcript is present in growing cells but was undetectable after 4 h of starvation. For comparison, *cAR1* gene expression was also assayed by RT-PCR. As expected, *cAR1* expression was strongly in-

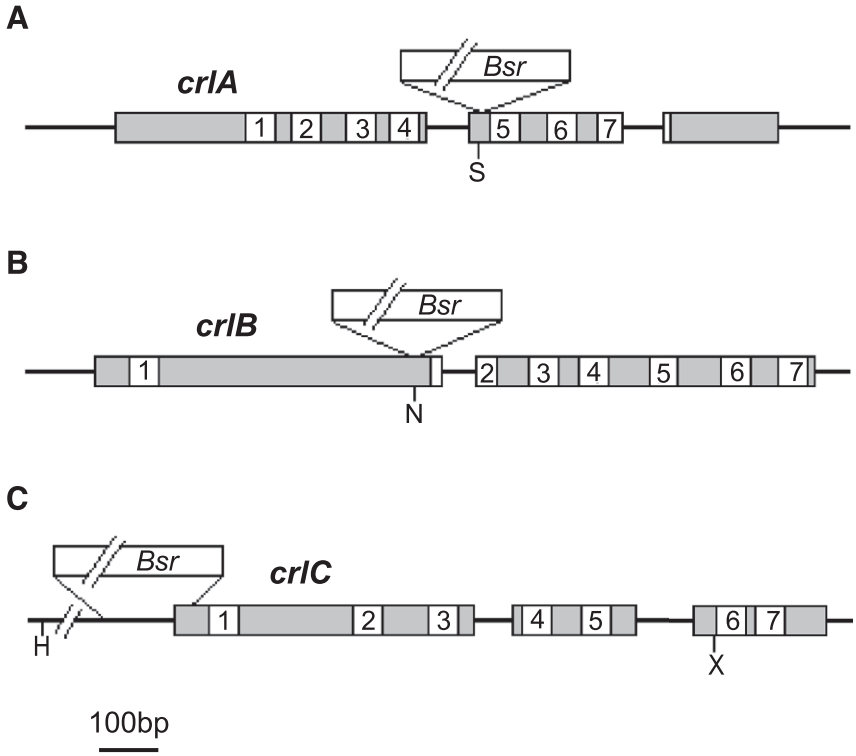


Fig. 4. Maps of *crlA–C* gene disruptions. (A) Map of the disrupted *crlA* locus disrupted by the *Bs*^{r} gene in either orientation. Digested genomic DNA of gene disruption mutants resulted in two 3.0-kb *Eco*RI–*Xho*I fragments and a 3.7- and 2.3-kb *Eco*RI–*Hin*dIII fragments as determined by genomic DNA blots hybridized with a *crlA* specific probe (data not shown). (B) Map of the *crlB* locus disrupted by the *Bs*^{r} gene. (C) Map of the *crlC* locus disrupted by the *Bs*^{r} gene. Disruption of the *crlB* and *crlC* loci was verified by PCR analysis. Exons of *crlA–C* genes are indicated by shaded bars and locations of predicted transmembrane domains are designated by numbered boxes. Location of *Bs*^{r} gene insertions and relevant restriction sites are indicated (S, *Spe*I; N, *Nco*I; H, *Hin*dIII; X, *Xba*I).

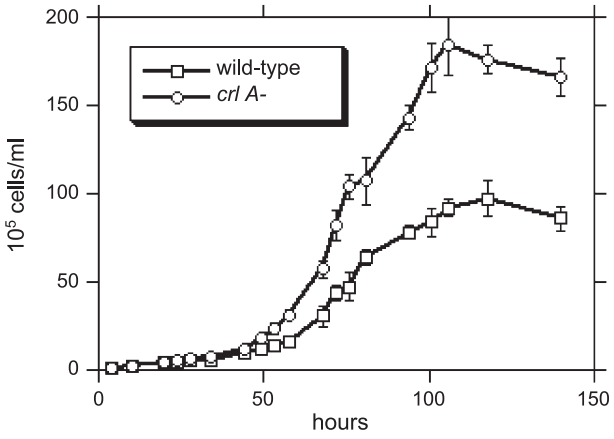


Fig. 5. Growth of *crlA*$^-$ mutants and wild-type cells. Cells were grown in shaking cultures of axenic medium as described in Materials and methods and cell counts were determined using a hemacytometer at the times indicated. Values represent the mean of four countings and error bars represent standard deviations. Several independent *crlA*$^-$ mutants were examined multiple times and similar results were obtained for each.

duced in early development, peaking after about 4 h. More RT-PCR product was obtained for *cAR1* transcript than either *crlB* or *crlC* despite having used 1/30th as much reverse transcription product as template. Thus, similar to *crlA*, the *crlB* and *crlC* genes appear to be expressed at relatively low levels.

Disruption of crl genes and chemotaxis analysis

To examine the potential functions of the Crl receptors, gene disruption mutants of each were created by the insertion of the *Bs*^{r} gene (Fig. 4). None of the *crl* mutants displayed grossly aberrant growth phenotypes in axenic medium or on bacterial lawns compared to wild-type cells, indicating none of the Crl receptors is essential for growth. However, axenic *crlA*$^-$ cultures reached cell densities nearly twice that of wild-type cultures before entering stationary phase (Fig. 5). The cell doubling time of *crlA*$^-$ cells was approximately 8 h during early exponential growth compared to the approximately 11-h doubling time of wild-type cells. To determine if this cell growth phenotype is cell autonomous, *crlA*$^-$ growth was analyzed in the presence of wild-type cells in mixed cultures with one of the strains labeled with GFP expression. GFP-labeled *crlA*$^-$ cells increased from 10% of the mixed culture population to 20% as cell growth reached stationary phase in the presence of wild-type cells indicating the *crlA*$^-$ growth phenotype is cell autonomous (Table 1). In mixed cultures containing mostly *crlA*$^-$ mutants, GFP-labeled wild-type cells dropped from 10% to less than 2% of the mixed population implying *crlA*$^-$ cells do not provide external factors that increase wild-type cell density.

All of the receptor mutants were tested for chemotaxis to cAMP and folic acid because these responses are dependent on GPCRs in *Dictyostelium*. Each receptor mutant displayed normal chemotaxis to cAMP as compared to wild-type cells (data not shown). This observation is consistent with the ability of the mutants to aggregate upon starvation. All of the receptor mutants displayed normal chemotaxis to folic acid as compared to wild-type cells indicating that responses to folic acid were not altered. The receptor mutants also exhibited normal plaque growth rates on bacterial lawns and exogenous folic acid inhibition of development as expected for cells responsive to folic acid (data not shown). In addition, all of the *crl* mutants exhibited normal folate-stimulated activation of the MAP kinase Erk2 (data not shown).

crlA$^-$ *mutants are delayed in aggregated tip formation*

Synchronized development of each *crl* mutant was analyzed by nutrient depletion and allowing cells to develop on non-nutrient agar plates. Development of the *crlB*$^-$ and *crlC*$^-$ mutants appeared similar to that of wild-type cells, suggesting the absence of any obvious morphological developmental defects (data not shown). However, *crlA*$^-$ mutants formed slightly larger aggregates that were delayed in the formation of the anterior tip (Fig. 6). After this delay of approximately 2 h, the mutant aggregates completed development with no other obvious delays or gross morphological phenotypes. Development processes before tip formation, such as the onset of aggregation, were also similar to that of wild-type cells.

The developmental delay of *crlA*$^-$ mutants could possibly result from inability of cells to generate or receive signals important for aggregate size and tip development. To determine if this developmental defect is cell-autonomous, as expected for a defect in signal reception, the

Table 1
Cell growth in chimeric populations

Chimeric population 9/1 ratio[a]	Chimeric population density[b]		GFP-labeled cell density[b]		% of GFP-labeled cells in chimeric population	
	(T = 2 h)	(T = 96 h)	(T = 2 h)	(T = 96 h)	(T = 2 h)	(T = 96 h)
Wild-type/*crlA*$^-$ (GFP)	1.7 ± 0.2	200 ± 1	0.13 ± 0.03	45 ± 2	8%	22%
crlA$^-$/wild-type (GFP)	1.7 ± 0.2	310 ± 2	0.18 ± 0.01	7.1 ± 0.9	10%	2%

[a] Chimeric populations were created by mixing unlabeled cells with GFP-labeled cells in a 9:1 ratio. Cells were grown in shaking cultures of axenic medium and cell densities were determined using a hemacytometer at the time indicated. Cells expressing the GFP were detected by fluorescence microscopy.
[b] Cell density values (× 10^5 cells/ml) are the mean of three experiments and the errors represent the standard deviations of the mean.

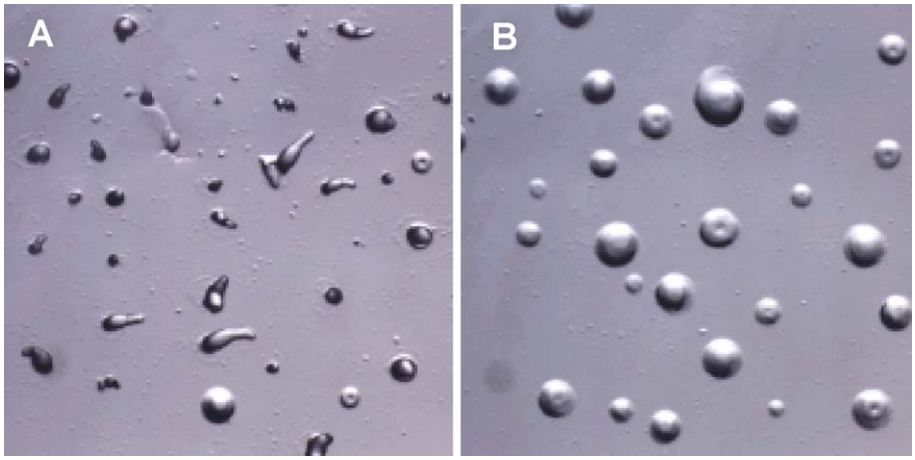


Fig. 6. Development of wild-type and *crlA*$^{-}$ mutants. Wild-type cells (A) and *crlA*$^{-}$ mutants (B) were grown and plated for development on non-nutrient agar as described in Materials and methods. Developmental morphology was photographed at 11 h after the onset of starvation.

receptor mutants were labeled using a GFP expression plasmid and mixed in a 1:20 ratio with unlabeled wild-type cells before development to create chimeric aggregates. After co-aggregating with the wild-type cells, the *crlA*$^{-}$ mutants were consistently underrepresented in the anterior prestalk cell region during later stages of development (Fig. 7). This heterogenous localization of the mutant cells in the chimeric aggregates was not affected by the GFP expression because the same GFP-labeled mutants were evenly distributed throughout chimeric organisms when they were mixed with unlabeled mutant cells.

Overexpression and complementation of crlA^{-} *gene function*

Increased or heterologous expression of signal transduction components can often lead to insights regarding the function of such components. To overexpress the *crlA* gene in both wild-type and *crlA*$^{-}$ cells, a genomic fragment was inserted into a G418-selectable integrating vector, which permits the selection of transformants acquiring multiple copies of the vector. Small colonies of G418 drug-resistant cells were observed after several days of selection at 5–10 μg/ml of G418 drug but the majority of these colonies disappeared by 10 days even when the selection was removed after 4 days. Cells transformed with empty vectors, lacking the *crlA* gene, were capable of continuous growth under identical conditions, suggesting that cells are intolerant of increased *crlA* gene expression. To test if the lethality of the *crlA* expression vectors resulted from *crlA* gene expression rather than just the presence of the vector, a frameshift mutation (*crlA*^{fs}) was introduced at the unique *Spe*I site in the *crlA* gene to prevent synthesis of a complete CrlA receptor. Wild-type and *crlA*$^{-}$ mutant cells containing the *crlA*^{fs} expression vector were capable of maintaining cell viability under higher G418 drug selection (5–10 μg/ml)

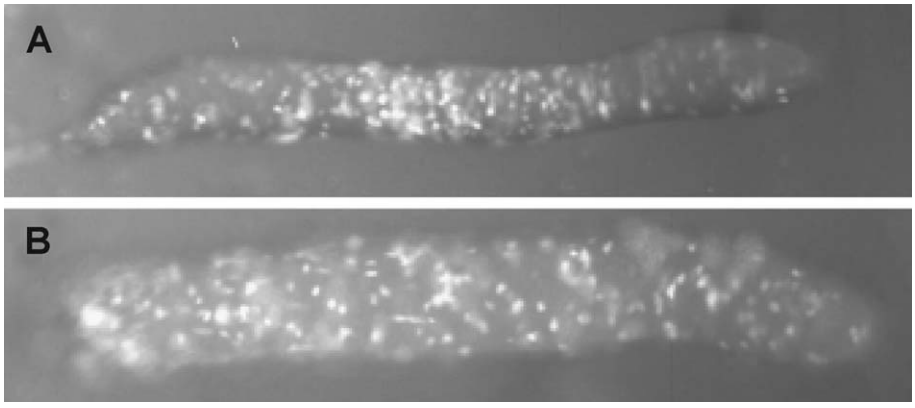


Fig. 7. Localization of GFP-expressing *crlA*$^{-}$ mutants in chimeric aggregates. *crlA*$^{-}$ mutant cells expressing GFP were mixed with wild-type (A) or unmarked *crlA*$^{-}$ mutant cells (B). All cells were grown and prepared for development as described in Materials and methods. Cells were mixed in a 1:20 ratio of GFP-expressing mutant cells to cells without GFP expression and then plated on non-nutrient agar for development. Developing aggregates were examined and photographed using fluorescence microscopy at 16 h after the onset of starvation. The anterior (prestalk) region of each slug-stage aggregate is on the right of each photograph.

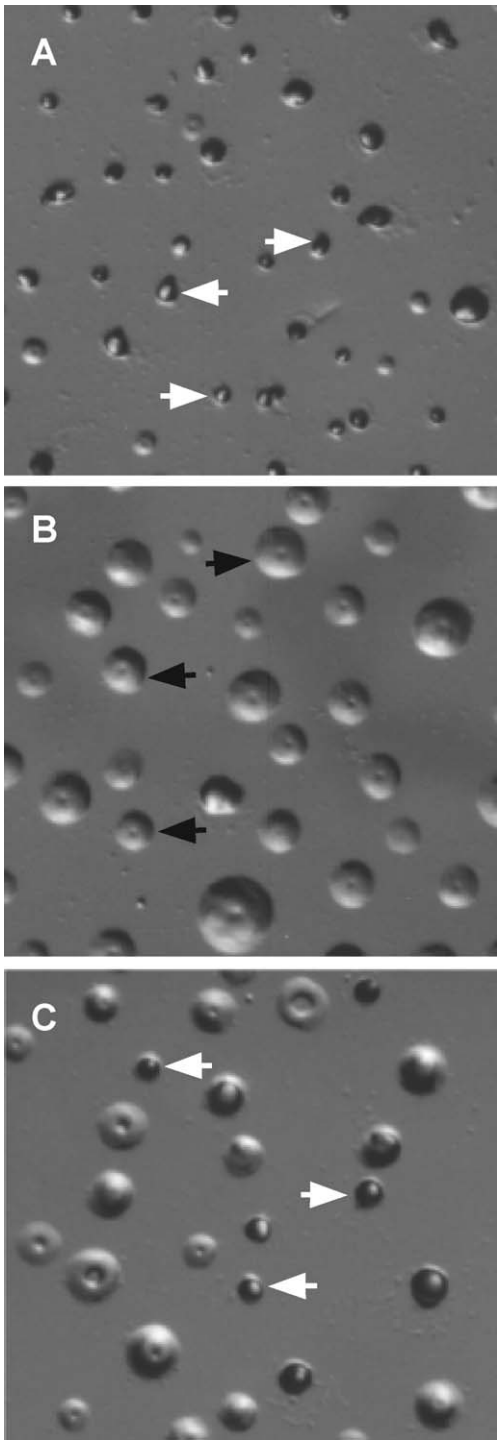


Fig. 8. Complementation of *crlA*$^-$ mutant developmental phenotypes. Wild-type (A), *crlA*$^-$ (B), and *crlA*$^-$ cells containing a *crlA* expression vector (C) were grown and developed on non-nutrient plates as described in Materials and methods. Developmental morphologies of aggregated cells were photographed at 11 h after nutrient depletion. Most aggregates of wild-type cells and some aggregates of *crlA*$^-$ cells containing a *crlA* expression vector displayed tipped mounds (representative examples indicated by white arrowheads), whereas many of the aggregates of *crlA*$^-$ cells lacking the expression vector remain flattened with apical indentions (representative examples indicated by black arrowheads).

than cells with the wild-type *crlA* expression vector, suggesting the translational product of the *crlA* gene is responsible for CrlA-associated lethality. Viable transformants containing wild-type *crlA* expression vectors with G418 drug resistance were obtained at a low efficiency by using reduced levels of drug selection (2–5 μg/ml), allowing cells with a low vector copy number to survive. Increased drug selection (5–10 μg/ml) on these transformants resulted in cell death. Viable transformants of wild-type cells were also obtained by introducing the wild-type *crlA* gene on a low-copy-number vector conferring resistance to the drug blasticidin, indicating cells can tolerate a limited level of vector-based *crlA* expression (data not shown).

The effects of *crlA* disruption and overexpression on growth are similar to those observed for the YakA protein kinase, suggesting this kinase might mediate signal transduction downstream from CrlA. To explore this possibility, we examined the ability of a *yakA*$^-$ mutation to suppress the cell death phenotype associated with overexpression of CrlA. Homologous recombination was used to create *yakA*$^-$ cells that were then transformed with *crlA* and *crlA*^{fs} expression vectors. While *yakA*$^-$ cells were notably more sensitive to G418 than were wild-type cells, more transformants were obtained with the *crlA*^{fs} mutant vector (approximately 3 transformants per 10 μg of vector) or an empty vector (no *crlA* sequences; approximately 5 transformants per 10 μg of vector) than with the wild-type *crlA* vector (approximately 0.2 transformants per 10 μg of vector) in side-by-side experiments implying *yakA*$^-$ cells are also sensitive to *crlA* expression.

The *crlA* expression vector was introduced into *crlA*$^-$ mutants and selection was conducted in medium containing low G418 concentrations (<5 μg/ml) to allow for the survival of low-copy-number transformants. Several transformants were examined for the complementation of the *crlA* gene disruption by analyzing the developmental morphology. All of the mutant clones with the *crlA* expression vector displayed a slightly accelerated rate in tip formation compared to the flattened mound phenotype observed for *crlA*$^-$ mutants without the vector after 11 h of nutrient depletion (Fig. 8). The *crlA* expression vector was not capable of completely rescuing the smaller aggregate size displayed by wild-type cells at this same time after nutrient depletion.

Discussion

The discovery of the three Crl receptors is consistent with previous studies that have predicted the existence of more GPCRs than the four cAMP receptors. The limited sequence identity of these receptors to each other and the four cAMP receptors suggests the Crl receptors represent one or more distinct classes of GPCRs but this distinction does not necessarily preclude these as cAMP receptors. The sequence similarities between the Crl and cAR receptors are spread over intracellular, extracellular, and transmembrane regions,

suggesting the Crl receptors might share structural or functional similarities (e.g., interactions with related signaling components) with the cAR receptors on either side of the plasma membrane. Both *crlA*$^-$ and *cAR2*$^-$ mutants are blocked in development before tip formation but *cAR2*$^-$ mutants terminate development at this stage, whereas the *crlA*$^-$ mutants resume development after the delay, indicating these receptors provide different functions with respect to this morphogenesis (Saxe et al., 1993). The phenotypic analyses of the *crl* mutants suggest the Crl receptors might function in pathways other than those stimulated by cAMP and folic acid (Table 2). The chemotaxis analysis indicates the *crlA*−*C* gene disruption mutants have normal cAMP chemotaxis and cAMP-mediated aggregation implying the CrlA−C receptors are not individually important for these cAMP responses. The receptors also do not appear to mediate responses to folate, including chemotaxis, inhibition of development, or MAP kinase activation, suggesting these receptors are not early-development folic acid receptors.

The cell growth phenotype of *crlA*$^-$ mutants in shaking cultures supports a role for CrlA function in the regulation of growth as nutrients become limited and/or cell concentrations increase. Such a phenotype could result from the inability of cells to sense external signals that might regulate cell division under these conditions. Other studies have indicated the presence of such a signal, designated as prestarvation factor (PSF), which promotes the expression of discoidin and other early developmental genes as nutrients become limited (Rathi et al., 1991). *Dictyostelium* cells produce another distinct signal called conditioned medium factor (CMF), a glycoprotein complex, which promotes cell aggregation as cell density increases (Gomer et al., 1991). However, preliminary studies suggest *crlA*$^-$ mutants respond to CMF (Raisley and Hadwiger, unpublished data). The elevated stationary phase cell density phenotype of *crlA*$^-$ mutants is similar to that of cells lacking the YakA protein kinase, an important regulator of *Dictyostelium*'s growth-to-development transition (Souza et al., 1998). Interestingly, YakA expression is induced in late-log stage cultures by a secreted factor, possibly PSF, and therefore might function downstream of the CrlA receptor in the regulation of cell cycle arrest at the onset of multicellular development. However, the inability of *yakA*$^-$ cells to survive the lethality of *crlA* overexpression indicates this lethality can occur independently of YakA function. A role for CrlA in cell cycle regulation is supported by evidence of similar functions of other GPCRs, including the related GCR1 receptor of *A. thaliana* (Colucci et al., 2002).

The aberrant development-associated *crlA*$^-$ mutants indicates a role for the CrlA receptor in establishing the size of aggregates and determining the onset of tip formation. While CrlA function does not affect cAMP chemotaxis or the onset of aggregation, this receptor might receive signals, such as CF (counting factor), known to regulate aggregate size (Brock and Gomer, 1999; Okuwa et al., 2001). Signals important for aggregate size and tip formation are likely to be interrelated as secondary or rival tip formation can divide large wild-type aggregates into smaller aggregates (Kopachik, 1982). Increases in aggregate size might also influence the timing of tip formation if cell signaling or cell sorting is delayed due the increased aggregate volume. Alternatively, alterations in prestalk cell differentiation could potentially delay tip formation by limiting the number or mobility of prestalk cells sorting to the tip (Early et al., 1995; Kopachik, 1982; Matsukuma and Durston, 1979; Ohmori and Maeda, 1986; Sternfeld and David, 1981; Traynor et al., 1992).

Both aggregate size and tip formation could also be pleiotropic responses to altered CrlA function during vegetative growth. Other *Dictyostelium* signaling components, such as the Gα4 and Gα5 subunits, are also expressed during vegetative growth and early development like the CrlA receptor, but neither component appears to be needed for development until the aggregate stage (Hadwiger and Firtel, 1992; Hadwiger et al., 1991, 1996). Many signaling

Table 2
Summary of *crlA*−*C* mutant properties

Mutant	Growth[a]	Development[b]	Co-development with wild type[c]	Folate-delayed development[d]	cAMP/folate chemotaxis[e]	cAMP-/folate-activated MAPK[f]
crlA$^-$	increased rate, density	delayed at mound stage	underrepresented in prestalk region	normal	+/+	both normal
crlB$^-$	normal	grossly normal	evenly distributed	normal	+/+	both normal
crlC$^-$	normal	grossly normal	evenly distributed	normal	+/+	both normal

[a] Cells were grown in axenic shaking cultures (*crlA*$^-$; see Fig. 5). Cell densities were determined in log and stationary phases with a hemacytometer.
[b] Mutant and wild-type control cells were developed on agar (*crlA*$^-$; see Fig. 6).
[c] Mutant cells expressing GFP (*crlA*$^-$; see Fig. 7) or β-galactosidase (*crlB*$^-$, *crlC*$^-$; data not shown) were co-mixed with unlabeled wild-type cells and developed on agar or filters. The distribution of labeled cells in slugs and fruiting bodies was ascertained by fluorescence microscopy and X-gal staining, respectively.
[d] Droplets (5 μl) containing 2×10^6 cells/ml were deposited on non-nutrient plates containing folic acid and development was compared to cells on plates without folic acid (data not shown).
[e] Droplets (approximately 5 μl) containing 2×10^6 cells/ml were deposited within 2-mm of similar droplets containing the indicated chemoattractant. Cells were scored as positive for chemotaxis if they accumulated at the side of the droplet nearest the chemoattractant after 30 min (cAMP) or 2 h (folate) (data not shown).
[f] Mitogen-activated protein kinase (MAPK) activated by cAMP or folate stimulation of cells was detected by immunoblotting with phospho-p44/42 MAPK-specific antibodies (Cell Signaling Technologies) (data not shown).

components with both vegetative and developmental expression, such as CrlA, Gα4 and Gα5, are not expressed specifically in prespore or prestalk cells but rather in cells that can be recruited to differentiate into a specific cell type later in development (Sternfeld and David, 1981, 1982; Van Driessche et al., 2002). A potential role for the CrlA receptor might be to regulate the cell cycle transition as cells starve and this could subsequently impact the development of specific cell types. A relationship between cell cycle progression and cell type-specific differentiation has been supported by studies that have monitored mitosis with respect to cell differentiation in isolated cells or in multicellular aggregates (Gomer and Firtel, 1987). Cells completing mitosis at the time of nutrient depletion tend to develop as prestalk cells, whereas those completing mitosis at other times develop as prespore cells. Therefore, altered cell division regulation in *crlA*$^-$ mutants might affect the ability of these cells to develop as prestalk cells.

The ability of *crlA*$^-$ mutants to eventually proceed through tip development indicates other mechanisms exist to overcome this temporary block in development. However, the scarcity of these mutant cells in the anterior prestalk region of chimeric aggregates indicates the mutant cells are not as competent as wild-type cells in forming anterior prestalk cells. The cell-autonomous nature of this defect in *crlA*$^-$ mutants is consistent with the role of CrlA as a receptor of external signals. Similar patterns of mutant cell distributions in chimeras have been reported for cells lacking the Gα5 G protein or the ERK1 protein kinase, suggesting these components might function in the same or related pathways (Gaskins et al., 1996; Natarajan et al., 2000). Similar to the *crlA*$^-$ mutant phenotype, the loss of Gα5 function also results in large aggregates with delayed tip formation (Hadwiger et al., 1996).

The *crlA* gene expression vectors can at least partially rescue the aberrant development associated with *crlA*$^-$ mutants but the apparent lethality associated with these vectors at high copy number indicates cells do not tolerate either increased or altered *crlA* gene expression. The cell death phenotype might possibly result from a CrlA receptor-mediated termination of vegetative growth if the receptor function is important for the transition from growth to development. A role for CrlA function in this transition is consistent with the increased cell number of *crlA*$^-$ mutants compared to wild-type cells if mechanisms exist to regulate cell division when nutrients become limited. Thus far, the CrlA receptor is the only known GPCR in *Dictyostelium* to affect cell viability when expressed from a vector.

The absence of obvious phenotypes in cells lacking either the CrlB or CrlC receptor during growth or development suggests that neither receptor provides essential functions for *Dictyostelium* cells under standard laboratory environments. The limited sequence identity between these receptors and the lack of other closely related receptors suggest these receptors do not provide redundant functions but perhaps perform functions required in environments or life cycles not yet tested or defined. These receptors might couple to known G proteins that also do not appear to be essential for growth or development under the conditions tested (Kumagai et al., 1991; Wu et al., 1994).

The four cAR and three Crl receptors cannot account for all of the GPCRs in *Dictyostelium* based on previous studies and therefore additional receptors are still likely to be found. Indeed, further scrutiny of the *Dictyostelium* cDNA and genome sequence databases revealed a family of at least six homologs of mammalian GPCRs for the neurotransmitter γ-aminobutyric acid or GABA (D. Hereld, unpublished observation). However, given the vast sequence diversity within the GPCR superfamily, a thorough accounting of *Dictyostelium* GPCRs awaits completion of the genome sequence and will likely require the use of search algorythms developed on quasi-periodic features, such as the seven transmembrane domain structure, which have proven useful for other sequenced genomes (Kim et al., 2000).

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